Sequential chromosome banding from single acetic acid fixed embryos of *Chromaphyosemion* killifishes (Cyprinodontiformes, Nothobranchiidae)

by

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ABSTRACT. - Chromosome preparation from fish embryos is a quick, cheap and simple method. However, it involves a fixation step in acetic acid, which has been reported to impair the suitability of chromosomes for certain banding techniques, and some available protocols require the mixing of several embryos to obtain preparations with a sufficient amount of metaphases, which can mask potential interindividual variation. Therefore, the method is used only rarely. Here, we describe a method for the preparation of a high number of high-quality metaphase chromosomes from single acetic acid fixed embryos of *Chromaphyosemion* killifishes and demonstrate that such preparations can be used routinely for conventional Giemsa staining, C-banding and sequential chromosome banding with chromomycin A_3, Giemsa and $AgNO_3$ or with DAPI, chromomycin A_3, Giemsa and C-banding, respectively. The protocol is particularly suitable for crossing experiments that require screening of various chromosome markers.

RÉSUMÉ. - Marquages chromosomiques séquentiels à partir d'embryons de *Chromaphyosemion* sp. (Cyprinodontiformes, Nothobranchiidae).

Préparer des chromosomes à partir d'embryons de poissons est une méthode peu onéreuse, simple et rapide. Cependant elle est peu utilisée, car elle nécessite une étape de fixation dans l'acide acétique qui est considérée comme impropre à l'application de certaines techniques de marquages en bandes. Par ailleurs, certains protocoles nécessitent de mélanger plusieurs embryons pour obtenir un nombre suffisant de métaphases, avec le risque de masquer de possibles variations chromosomiques interindividuelles. La méthode décrite ici permet de préparer un nombre élevé de métaphases de bonne qualité à partir d'un seul embryon de *Chromaphyosemion*. Elle démontre que de telles préparations peuvent être réalisées en routine pour appliquer séquentiellement sur les chromosomes des marquages successifs tels que Chromomycine A_3, Giemsa, nitrate d'argent ou bien DAPI, Chromomycine A_3, Giemsa et bandes C respectivement. Ce protocole est particulièrement intéressant dans les expériences de croisement qui nécessitent la comparaison de marqueurs chromosomiques de types différents.

Key words. - Nothobranchiidae - *Chromaphyosemion* - Fish cytogenetics - Embryos - Crossing experiments - Chromomycin A_3 - C-banding - $AgNO_3$ - DAPI.

Over the past decades, a considerable number of methods for the preparation of fish chromosomes have been developed (reviews e.g. Denton, 1973; Ojima, 1982; Gold *et al*., 1990; Klinkhardt, 1991; Ozouf-Costaz *et al*., 1992). Widely used methods include direct preparation from kidney (a haematopoietic tissue in fishes), cell cultures from fin biopsies and lymphocyte cultures (Fujiwara *et al*., 2001). All methods have particular advantages and disadvantages. Direct preparation of chromosomes from kidney is comparatively simple, quick and cheap. However, in fishes homeostasis is strongly affected by seasonal effects. Therefore, though methods for mitotic stimulation in vivo are available (e.g. Ojima and Kurishita, 1980; Cucchi and Baruffaldi, 1989; Cucchi and Baruffaldi, 1990), it is not always possible to obtain preparations with a sufficient number of high-quality metaphases. Besides, the fish have to be sacrificed for chromosome preparation, which can be a problem if the specimens are hard to get and must be deposited in museum collections and/or shall be used in additional experiments. Cell cultures from fin biopsies do not necessitate sacrificing the fish and usually provide a higher amount of high-quality metaphases than direct preparation from kidney, but this method is comparatively expensive and time-consuming. Lymphocyte cultures yield a high number of high-quality metaphase chromosomes but require around 0.5 ml of blood, which is difficult to get from small specimens.

An alternative method is the preparation of chromosomes from fish embryos (e.g. Denton, 1973; Phillips *et al*., 1985; Chourrout and Happe, 1986; Baksi and Means, 1988; Ueda *et al*., 1991; Klinkhardt, 1992). This method is quicker and cheaper than cell cultures and does not require the sacrifice of adult specimens. Despite these advantages, the method is used only rarely. To date, its application has been limited to salmonids (e.g. Phillips *et al*., 1985; Chourrout and Happe, 1986; Inokuchi *et al*., 1994; Fujiwara *et al*., 1998), bitterlings (e.g. Ueda *et al*., 1997; Ueda *et al*., 2001) and only a few species from other fish groups (Baksi and Means, 1988; Ueda and Naoi, 1999). Presumably, one reason for this is

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that some protocols (e.g., Klinkhardt, 1992) require the mixing of several embryos to obtain preparations with a sufficient number of metaphases, which conceals potential individual variation and thus renders these protocols unsuitable for examinations in which chromosomal variability between individuals is to be expected. A further reason might be that the method involves mincing the embryonal tissues in 50-60% acetic acid to form a single cell suspension. According to several reports (Holmquist and Motara, 1987; Sumner, 1990 and references therein), the acetic acid removes certain chromosomal components from the chromatin and thus somehow lowers the suitability of the chromosomes for certain staining and banding techniques. Correspondingly, only very few papers report the successful use of chromosome preparations from acetic acid treated fish embryos for chromosome banding techniques (e.g., Phillips *et al*., 1985), and to the best of our knowledge, no protocol for sequential chromosome banding using such preparations has been published.

The cytogenetic study of karyotypically diverse organisms such as nothobranchiid killifishes requires a fast and efficient method for chromosome preparation from single specimens, which allows the examination of a large number of individuals and the application of sequential banding techniques. However, in our study of karyotype evolution in *Chromaphyosemion* killifishes, chromosome preparation from head kidney (Ráb, 1981) did not yield a sufficient amount of metaphases of good quality, not even when combined with methods for the stimulation of mitotic activity (Cucchi and Baruffaldi, 1989; Cucchi and Baruffaldi, 1990). Thus, the objectives of the present study were (i) to develop an optimised method for simple, quick and cheap chromosome preparation from single killifish embryos, and (ii) to test whether these preparations were suitable for sequential chromosome banding with the banding and staining techniques most widely used in fish cytogenetics (fluorescence banding with chromomycin A_3 (CMA_3) and 4'6-diamidino-2-phenylindole (DAPI), C-banding, $AgNO_3$-staining, conventional Giemsa staining).

MATERIALS AND METHODS

Experimental animals

A total of 138 specimens belonging to 21 populations of 8 described and 2 tentative *Chromaphyosemion* species were analyzed (Tab. I). The fishes were bred at the Institut für Evolutionsbiologie and Ökologie in Bonn, Germany, according to the method outlined by Scheel (1968). Eggs were collected daily and kept in small plastic basins with aquarium water (23°C, pH 7) until chromosome preparation was carried out.

Chromosome preparation

Chromosome preparation followed the principle described by Chourrout and Happe (1986). Some modifications were necessary to ensure a consistently high quality of the preparations. Since it is known that embryos of different age show different mitotic rates (Denton, 1973; Chourrout and Happe, 1986), we used embryos ranging from 2 to 8 days of age. In order to determine the optimum incubation time and the optimum colchicine concentration for mitostatic treatment, whole eggs were incubated in 0.02% colchicine (Fluka 27650) for 8-16 h or in 0.05% colchicine for 4-6 h at 28°C. The colchicine solution was prepared with aquarium water. Following mitostatic treatment, the embryos were dissected out of the eggs in a watchglass filled with 0.9% NaCl and individually transferred to 15 ml centrifuge

Table I. - Number of specimens per species/population examined with sequential CMA_3-banding, Giemsa staining and $AgNO_3$-staining (method A), sequential DAPI-banding, CMA_3-banding, Giemsa staining and C-banding (method B), C-banding only (method C) and Giemsa staining only (method D). For methods A and C, slides from the same specimens were used. Thus, the total number of specimens analysed is 138. *[Nombre d'individus par espèce/population examinés par marquage séquentiel au* CMA_3*, coloration au Giemsa et à l'*$AgNO_3$ *(méthode A), par marquage séquentiel DAPI,* CMA_3*, coloration au Giemsa et marquage C (méthode B), marquage C seul (méthode C) et coloration Giemsa seule (méthode D). Pour les méthodes A et C, des coupes des mêmes spécimens ont été utilisées. Ainsi, le nombre total de spécimens analysés est de 138.]*

	No. of specimens examined with method			
Species, population	A	B	C	D
C. alpha "GJS00/34"	3	2	2	3
C. bivittatum "Funge"	4	3	2	3
C. bivittatum "KV03/32"	4	2	2	3
C. bivittatum "KV03/33"	4	2	2	3
C. bivittatum "KV03/34"	4	2	2	3
C. bivittatum "KV03/35"	4	2	2	3
C. kouamense "G02/115"	3	2	2	3
C. loennbergii "KEK98/7"	2	-	2	3
C. lugens "KV03/40"	4	3	2	3
C. melanogaster "KEK98/6"	2	-	2	3
C. poliaki "C03/36"	2	-	2	4
C. poliaki "C03/42"	2	-	2	3
C. poliaki "C03/42"	2	-	2	3
C. splendopleure "C03/01"	2	-	2	3
C. splendopleure "C03/14"	2	-	2	3
C. splendopleure "C03/46"	2	-	2	3
C. cf. *splendopleure* "Lykoko"	2	-	2	3
C. cf. *splendopleure* "C03/41"	2	-	2	3
C. sp. aff. *splendopleure* "CMM8"	2	-	2	3
C. sp. aff. *splendopleure* "Bioko"	2	-	2	3
C. sp. aff. *splendopleure* "C03/44"	2	-	2	3
Total	56	18	42	64

tubes, containing 5 ml of 0.8% trisodium citrate ($C_6H_5Na_3O_7$) or 5 ml of 0.075 M KCl each. For hypotonic treatment, we tested incubation times ranging from 25-90 min at 23°C. After hypotonic treatment, the hypotonic solution was replaced by 5 ml of freshly prepared ice-cold methanol: acetic acid (3:1) and the embryos were fixed for 20 min at 4°C. This fixation step was repeated twice. Subsequently, the preparations were stored overnight in closed tubes with fresh fixative at 4°C. On the next day, each embryo was minced individually in 50 μl of 50% acetic acid for 30 s, using a 1.5 ml Eppendorf tube and a micropestle. Immediately prior to slide preparation, SuperFrost slides were cleaned by incubation in ethanol with 1% HCl for 20 min at room temperature, wiped dry with a paper towel and heated to about 46°C on a slide warmer. Using a micropipette, the cell suspension was dropped onto a slide, left there for 20 seconds and sucked back into the micropipette, leaving a ring of cells on the slide. This procedure was repeated twice, creating three adjacent rings per slide. The slides were left to dry on the slide warmer for 2-3 min. Three slides were prepared from one embryo.

Mitotic indices and the proportion of analysable metaphases relative to the total amount of metaphases were estimated for two species (*C.* cf. *splendopleure* "C03/41" and *C. loennbergii* "KEK98/7") by counting the total number of nuclei, the total number of metaphases and the number of analysable metaphases in a transect leading through the middle of the 3 rings of cells, using low magnification (200x). Per species, mitotic indices and the proportion of analysable metaphases were determined for 3 specimens aged 3 days and 3 specimens aged 6 days at the time of preparation.

Chromosome banding

Preparations from at least 3 individuals per population were stained in buffered Giemsa (5%, pH 6.88, 10 min) in order to determine number and morphology of chromosomes. Additionally, at least 2 slides from different individuals per population were used for sequential banding with CMA_3, Giemsa and $AgNO_3$, and a second set of slides from the same specimens was used for C-banding. In 8 populations belonging to 4 different species, sequential chromosome banding with DAPI, CMA_3, Giemsa and C-banding was carried out as well, using at least 2 slides from 2 specimens per population. Table I gives details on the species, the populations and the number of specimens examined with different banding and staining techniques. CMA_3-banding and $AgNO_3$-staining were performed according to Sola *et al.* (1992) and Howell and Black (1980), respectively. DAPI-banding was carried out with and without vectashield. DAPI-banding without vectashield followed Sola *et al.* (1992), with minor modifications. Slides were preincubated in McIlvaine's buffer with 10 mM $MgCl_2$ for 10 min, stained with methyl green (35 mg methyl green / 100 ml McIlvaine's buffer) for 15 min, washed in McIlvaine's buffer, stained with DAPI solution (0.5 μg DAPI / 1 ml McIlvaine's buffer) for 20 min, washed again in McIlvaine's buffer and mounted in 50% glycerol. DAPI staining with vectashield was simply done by dropping two drops of vectashield mounting medium with DAPI (Vector Laboratories) onto a slide followed by sealing with a cover slip in the dark. The fluorescence was observed after 15 minutes of treatment. C-banding followed the method of Haaf and Schmid (1984) with some modifications. Slides were treated with 0.2 N HCl at room temperature for 10 min, rinsed in distilled H_2O and allowed to dry partially for 15-20 min. Subsequently, they were incubated in a saturated $Ba(OH)_2$ solution at 33°C for 4 min, rinsed instantly in 0.2 N HCl and distilled H_2O, incubated in 2xSSC at 65°C for 2 h and rinsed with distilled H_2O. After the slides had dried, they were stained in buffered Giemsa (5%, pH 6.88, 10 min).

For sequential chromosome banding, specific destaining procedures were employed. After removing the cover slip, CMA_3-banded slides were incubated in xylene for 2 min and air-dried. Subsequently, the slides were incubated in benzine (SIGMA 12270) for 2 min, air-dried and incubated in A. dest. for 10 min. After partial air-drying for 15 min, the incubation steps in xylene, benzine and A. dest were repeated three times. Finally, the slides were air-dried for at least one hour and incubated in 70% ethanol (prepared from absolute ethanol) for 3 min, rinsed with A. dest. and air-dried. Slides treated in this fashion were suitable for Giemsa staining,

Table II. - Mitotic indices and proportion of analysable metaphases in preparations of 3-days and 6-days old embryos of *C.* cf. *splendopleure* "C03/41" and *C. loennbergii* "KEK98/7". Per species and age-group, 3 specimens were analysed. *[Indices mitotiques et proportions de métaphases analysables dans des préparations d'embryons de* C. *cf.* splendopleure *"CO3/41" et* C. loennbergii *"KEK98/73" de 3 et 6 jours. Trois spécimens ont été analysés par espèce et par groupe d'âge.]*

	C. cf. *splendopleure* "C03/41"		*C. loennbergii* "KEK98/7"	
Embryo age (days)	3	6	3	6
Mitotic index (%), mean	2.83	1.01	2.89	1.05
Mitotic index (%), range	2.33-3.57	0.64-1.32	2.04-3.81	0.39-1.44
Proportion of analysable metaphases (%), mean	35.1	26.0	25.2	31.8
Proportion of analysable metaphases (%), range	31.9-39.4	25.0-28.0	20.0-28.3	22.0-50.0

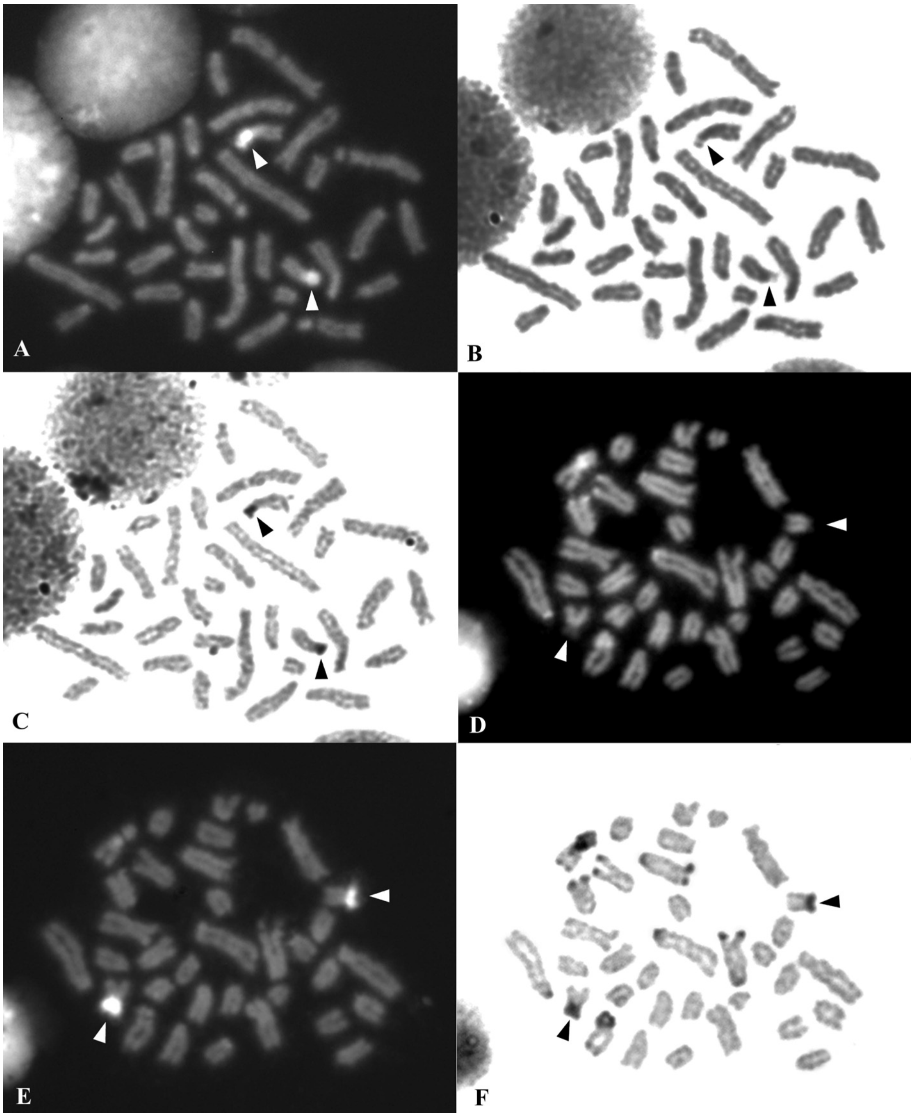

Figure 1. - Metaphase cells of *Chromaphyosemion bivittatum* "KV03/34" prepared from single embryos. **A-C**: Sequential banding of the same metaphase with (**A**) CMA_3, (**B**) Giemsa and (**C**) $AgNO_3$. **D-F**: Sequential banding of a metaphase cell from a different specimen with (**D**) DAPI, (**E**) CMA_3, Giemsa (not shown) and (**F**) C-banding. Arrowheads indicate the NOR-bearing chromosome pair. The NORs are GC-rich (**A, E**) and C-positive (**F**). C-positive heterochromatin contains AT-rich sequences as evidenced by both DAPI-positive and C-positive signals (**D, F**) and GC-rich sequences as indicated by both CMA_3-positive and C-positive signals (**E, F**). *[Métaphases de* Chromaphyosemion bivittatum *"KV03/34" préparées à partir d'un seul embryon.* ***A-C*** *: Marquages chromosomiques séquentiels de la même métaphase avec (**A**)* CMA_3*, (**B**) Giemsa et (**C**)* $AgNO_3$*.* ***D-F*** *: Marquages chromosomiques séquentiels d'une métaphase d'un spécimen différent avec (**D**) DAPI, (**E**)* CMA_3*, Giemsa (absent) et (**F**) marquage C. Les têtes des flèches indiquent la paire de chromosomes portant NOR. Les NORs sont riches en GC (**A, E**) et positifs C (**F**). Les hétérochromatines positives C contiennent des séquences riches en AT comme démontré à la fois par les signaux positifs DAPI et C, et des séquences riches en GC comme indiqué par les signaux positifs* CMA_3 *et C (**E, F**).]*

$AgNO_3$-staining and C-banding. Giemsa-stained slides, which were investigated without cover slip, were cleaned from immersion oil by treatment with xylene, benzine and A. dest. as described above and destained by incubation in freshly prepared, cold fixative for 2-3 min, followed by air-drying. These slides could be used for $AgNO_3$-staining and C-banding. DAPI-banded slides were rinsed in A. dest. and air-dried and were suitable for all banding and staining techniques tested including CMA_3-banding.

RESULTS AND DISCUSSION

The optimum age for chromosome preparation was determined to be 3-5 days after fertilisation. Two-days embryos had high mitotic indices but provided not enough cell material for good preparations. Three-days embryos showed a sufficient amount of cell material and still quite high mitotic indices, with embryos of *C.* cf. *splendopleure* "Muyuka" and *C. loennbergii* "KEK98/7" having mean mitotic indices of 2.83% and 2.89%, respectively (Tab. II). In six-days embryos of the same species, mean mitotic indices decreased to 1.01% and 1.05%, respectively (Tab. II). Regardless of the age of the embryos examined, about 30% of all metaphases were of sufficient quality for karyotype analysis (Tab. II). The high mitotic index during the first days of embryonic development was probably due to tail bud formation and growth. With the beginning of the eyed stage, the mitotic index started to decrease. This finding agrees with previous reports about the optimum developmental stage of rainbow trout embryos for chromosome preparation (Chourrout and Happe, 1986). Taken together, these results suggest that this embryonic stage is generally the most suitable for chromosome preparation.

The optimum mitostatic treatment consisted of incubation in 0.02% colchicine for 8-9 h or in 0.05% colchicine for 4-5 h. Both treatments yielded approximately equal amounts of metaphases with optimally contracted chromosomes and well paired chromatids (Fig. 1). The examined populations showed only very little variability in their response to mitostatic treatment.

With respect to optimum hypotonic treatment, variability between species and in some cases also between populations of the same species was more marked, indicating a certain degree of cytological differentiation. Depending on the population under investigation, good spreading of chromosomes could be achieved using 0.8% trisodium citrate for 40-60 min or 0.075 M KCl for 35-50 min. Generally, 0.075 M KCl yielded a faster spreading than 0.8% trisodium citrate while at the same time resulting in more scattering and a visibly higher contraction of chromosomes. The experimentally determined optimum mitostatic and hypotonic treatment differed slightly from the optimum procedures for rainbow trout embryos as described by Chourrout and Happe (1986). This finding suggests that the application of this protocol to other fish groups than killifishes will probably require minor modifications of these experimental steps to produce satisfactory results.

All preparations were suitable for conventional Giemsa staining, C-banding and sequential banding with CMA_3, Giemsa and $AgNO_3$ (Fig. 1A-C). Sequential banding with CMA_3 and $AgNO_3$ showed good correspondence of CMA_3-positive and Ag-positive NORs (Fig. 1A, B). In the 8 populations examined with this method, sequential banding with DAPI, CMA_3, Giemsa and C-banding was possible as well (Fig. 1D-F). It is important to note that the method used for DAPI-banding had a significant effect on the quality of subsequent bandings and stainings. DAPI with Vectashield resulted in rather swollen chromosomes and adversely affected chromosome morphology, while no such effect was observed when using DAPI without vectashield. Contrary to previous reports about an impaired suitability of acetic acid fixed tissue for chromosome banding techniques (Holmquist and Motara, 1987; Sumner, 1990 and references therein), our results show that chromosomes from acetic acid fixed fish embryos prepared according to the present protocol could be used for all chromosome banding techniques tested here without any problem. This finding confirms previous results about the suitability of such preparations for several banding techniques (Phillips *et al.*, 1985). Furthermore, the present study demonstrates for the first time that these preparations can be routinely used for sequential application of the banding and staining techniques most widely used in fish cytogenetics and provides a detailed protocol for this methodology. This point is particularly important since fish chromosomes do not normally show euchromatic, G-type banding patterns (Medrano *et al.*, 1988; Sumner, 1990). Therefore, the use of sequential banding methods is necessary in order to identify the homologous chromosomes within the karyotype of a given species and to determine chromosomal rearrangements that distinguish the karyotypes of different species. Although not tested here, studies performed on chromosome preparations from acetic acid treated embryos of salmonids suggest that such material is also suitable for fluorescence in situ hybridisation (FISH) (Fujiwara *et al.*, 1998).

Besides, it is important to note that contrary to some other protocols (e.g. Klinkhardt, 1992), the present technique permits chromosome preparation from single individuals and thus is suitable for the investigation of karyotypic variability between individuals.

In summary, our modification of the method of Chourrout and Happe (1986) permitted the preparation of a high number of high-quality metaphases suitable for the sequential application of the most widespread staining and banding techniques from single acetic acid fixed embryos of *Chromaphyosemion* killifishes. The method is considerably faster, cheaper and simpler than cell culturing techniques and yielded by far more high-quality metaphases than direct preparation from head kidney. With minor modifications of the mitostatic and hypotonic treatment, it will probably produce good results also with species from different fish groups. Since no centrifugation steps are needed, it requires only very simple laboratory equipment and therefore allows cytogenetic analyses of fishes to be carried out even in laboratories not specifically equipped for this purpose. Unlike widely used methods for the direct preparation of chromosomes from kidney, chromosome preparation from embryos does not require the sacrifice of adult specimens which is especially important if the fishes under investigation are hard to obtain and needed for additional experiments. Apart from standard karyotype characterisation, possible applications of the method include the fast and efficient analysis of offspring karyotypes from crossbreeding experiments and the assessment of the effect of genotoxic agents on early life stages of fish.

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